The present invention relates to new porcine circovirus (PCV for *Porcine CircoVirus)* strains responsible for the PMWS syndrome (*Porcine Multisystemic Wasting Syndrome* also called *Post-Weaning Multisystemic Wasting Syndrome*) to reagents and methods allowing their detection, to methods of vaccination and to vaccines, as well as to methods of producing these reagents and vaccines.

PCV was originally detected as a noncytopathogenic contaminant in pig kidney cell lines PK/15. This virus was classified among the Circoviridae with the chicken anaemia virus (CAV for *Chicken Anaemia Virus*) and the PBFDV virus (*Pscittacine Beak and Feather Disease Virus*). It is a small nonenveloped virus (from 15 to 24 nm) whose common characteristic is to contain a genome in the form of a circular single-stranded DNA of 1.76 to 2.31 kb. It was first thought that this genome encoded a polypeptide of about 30 kDa (Todd et al., Arch Virol 1991, 117; 129-135). Recent work has however shown a more complex transcription (Meehan B. M. et al., 1997, 78; 221-227). Moreover, no significant homologies in nucleotide sequence or in common antigenic determinants are known between the three types of circoviruses known.

The PCV derived from the PK/15 cells is considered not to be pathogenic. Its sequence is known from B.M. Meehan et al., J. Gen. Virol 1997 (78) 221-227. It is only very recently that some authors have thought that strains of PCV could be pathogenic and associated with the PMWS syndrome (Gupi P.S. Nayar et al., Can. Vet. J, vol. 38, 1997: 385-387 and Clark E.G., Proc. Am. Assoc. Swine Prac. 1997; 499-501). Nayar et al. have detected PCV DNA in pigs having the PMWS syndrome using PCR techniques. No wild-type PCV strain has however been isolated and purified so far.

The PMWS syndrome detected in Canada, the United States and France is clinically characterized by a gradual loss of weight and by manifestations such as

tachypnea, dyspnea and jaundice. From the pathological point of view, it is manifested by lymphocytic or granulomateus infiltrations, lymphadenopathies and, more rarely, by hepatitis and lymphocytic or granulomateus nephritis (Clark E.G., Proc. Am. Assoc. Swine Prac. 1997; 499-501; La Semaine Vétérinaire No. 26, supplement to La Semaine Vétérinaire 1996 (834); La Semaine Vétérinaire 1997 (857): 54; Gupi P.S. Nayar et al., Can. Vet. J, vol. 38, 1997; 385-387).

The applicant has succeeded in isolating five new PCV strains from pulmonary or ganglionic samples obtained from farms situated in Canada, the United States (California) and France (Brittany), hereinafter called circoviruses according to the invention. These viruses have been detected in lesions in pigs with the PMWS syndrome, but not in healthy pigs.

The applicant has, in addition, sequenced the genome of four of these strains, namely the strains obtained from Canada and the United States as well as two French strains. The strains exhibit a very strong homology with each other at the nucleotide level, exceeding 96% and much weaker with the PK/15 strain, about 76%. The new strains can thus be considered as being representative of a new type of porcine circovirus, called here type II, type I being represented by PK/15.

The subject of the present invention is therefore the group II porcine circovirus, as defined above, isolated or in the form of a purified preparation.

The invention relates to any porcine circovirus capable of being isolated from a physiological sample or from a tissue sample, especially lesions, from a diseased pig having the PMWS syndrome, especially following the method described in the examples, in particular type II circovirus.

The subject of the present invention is more particularly purified preparations of five strains, which were deposited at the ECACC (European Collection

of Cell Cultures, Centre for Applied Microbiology & Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom) on Thursday 2 October 1997:

- accession No. V97100219 (called here Imp. 1008PCV)
- accession No. V9700218 (called here Imp. 1010PCV)
- accession No. V97100217 (called here Imp. 999PCV),

and, on Friday 16 January 1998:

- accession No. V98011608 (called here Imp. 1011-48285)
- accession No. V98011609 (called here Imp. 1011-48121).

The invention aims to consider the porcine circoviruses isolated from a diseased pig and/or the circoviruses having a significant serological similarity with the strains of the invention and/or the circoviruses having cross-hybridization with the strains of the invention under stringency conditions such that there is no hybridization with the PCV PK/15 strain.

The viral strains isolated from a physiological sample or from a tissue sample, especially a lesion, from a pig having the PMWS syndrome can be advantageously propagated on cell lines such as especially pig kidney cell lines, in particular PK/15 cells free from contamination (in particular for PCV, as well as for pestiviruses, porcine adenoviruses and porcine parvoviruses) for their multiplication or specifically for the production of antigen, whole (e.g. virus) and/or subunits (e.g. polypeptides).

Very remarkably and unexpectedly, these isolates have proved very productive in culture on PK/15 cells, which have undeniable advantages for the production of virus or antigen, in particular for the production of inactivated vaccine.

The subject of the present invention is also the preparations of circoviruses isolated after

passages on cells, especially cell lines, e.g. PK/15 cells, cultured in vitro while being infected with at least one of the circoviruses according to the invention or of any porcine circovirus capable of being isolated form a physiological sample or from a tissue sample, especially lesions, from a pig having the PMWS syndrome. Its subject is also the culture extract or supernatant, optionally purified by standard techniques, and in general any antigenic preparation obtained from in vitro cultures.

The subject of the invention is also the immunogenic active ingredients and the vaccines containing at least one antigen as defined above.

They may be immunogenic active ingredients based on attenuated live whole viruses, or vaccines prepared with these active ingredients, the attenuation being carried out according to the customary methods, e.g. by passage on cells, preferably by passage on pig cells, especially lines, such as PK/15 cells (for example from 50 to 150, especially of the order of 100, passages). These vaccines comprise in general a vehicle or diluent acceptable from the veterinary point of view, optionally an adjuvant acceptable from the veterinary point of view, as well as optionally a freeze-drying stabilizer.

These vaccines will preferably comprise from 10^3 to 10^6 TCID50.

They may be immunogenic active ingredients or vaccines based on circovirus antigen according to the invention, in an inactivated state. The vaccine comprises, in addition, a vehicle or a diluent acceptable from the veterinary point of view, with optionally in addition an adjuvant acceptable from the veterinary point of view.

The circoviruses according to the invention, with the fractions which may be present, are inactivated according to techniques known to persons skilled in the art. The inactivation will be preferably carried out by the chemical route, e.g. by exposing the

antigen to a chemical agent such as formaldehyde (formalin), paraformaldehyde, β-propiolactone or ethyleneimine or its derivatives. The preferred method of inactivation will be herein the exposure to a chemical agent and in particular to ethyleneimine or to β-propiolactone.

Preferably, the inactivated vaccines according to the invention will be supplemented with adjuvant, advantageously by being provided in the form of emulsions, for example water-in-oil or oil-in-water, according to techniques well known to persons skilled in the art. It will be possible for the adjuvant character to also come from the incorporation of a customary adjuvant compound into the active ingredient.

Among the adjuvants which may be used, there may be mentioned by way of example aluminium hydroxide, the saponines (e.g. Quillaja saponin or Quil A; see Vaccine Design, The Subunit and Adjuvant Approach, 1995, edited by Michael F. Powel and Mark J. Newman, Plennum Press, New-York and London, p.210), Avridine® (Vaccine Design p. 148), DDA (Dimethyldioctadecyl-ammonium bromide, Vaccine Design p. 157), Polyphosphazene (Vaccine Design p. 204), or alternatively oil-in-water emulsions based on mineral oil, squalene (e.g. SPT emulsion, Vaccine Design p. 147), squalene (e.g. MF59, Vaccine Design p. 183), or water-in-oil emulsions based on metabolizable oil (preferably according to WO-A-94 20071) as well as the emulsions described in US-A-5,422,109. It is also possible to choose combinations of adjuvants, for example Avridine® or DDA combined with an emulsion.

These vaccines will preferably comprise from 10^6 to 10^8 TCID50.

The live vaccine adjuvants can be selected from those given for the inactivated vaccine. The emulsions are preferred. To those indicated for the inactivated vaccine, there may be added those described in WO-A-9416681.

As freeze-drying stabilizer, there may be mentioned by way of example SPGA (Bovarnik et al., J. Bacteriology 59, 509, 950), carbohydrates such as sorbitol, mannitol, starch, sucrose, dextran or glucose, proteins such as albumin or casein, derivatives of these compounds, or buffers such as alkali metal phosphates.

The applicant has, in addition, obtained the genome of four of the isolates, identified SEQ ID NO: 1 to 4 and optionally 6.

The subject of the present invention is therefore a DNA fragment containing all or part of one of these sequences. It goes without saying that the invention automatically covers the equivalent sequences, that is to say the sequences which do not change the functionality or the strain-specificity of the sequence described or of the polypeptides encoded by this sequence. There will of course be included the sequences differing by degeneracy of the code.

The invention also covers the equivalent sequences in the sense that they are capable of hybridizing with the above sequence under high stringency conditions and/or have a high homology with the strains of the invention and belong to group II defined above.

These sequences and their fragments can be advantageously used for the in vitro or in vivo expression of polypeptides with the aid of appropriate vectors.

In particular, the open reading frames, forming DNA fragments according to the invention, which can be used to this effect have been identified on the genomic sequence of the type II circoviruses. The invention relates to any polypeptide containing at least one of these open reading frames (corresponding amino acid sequence). Preferably, the invention relates to a protein essentially consisting of ORF4, ORF7, ORF10 or ORF13.

For the expression of subunits in vitro, as a means of expression, E. coli or a baculovirus will be preferably used (US-A-4,745,051). The coding sequence(s) or their fragments are integrated into the baculovirus genome (e.g. the baculovirus Autographa californica Nuclear Polyhedrosis Virus AcNPV) and the latter is then propagated on insect cells, e.g. Spodoptera frugiperda Sf9 (deposit ATCC CRL 1711). The subunits can also be produced in eukaryotic cells such as yeasts (e.g. Saccharomyces cerevisiae) or mammalian cells (e.g. CHO, BHK).

The subject of the invention is also the polypeptides which will be produced in vitro by these expression means, and then optionally purified according to conventional techniques. Its subject is also a subunit vaccine comprising at least one polypeptide as thus obtained, or fragment, in a vehicle or diluent acceptable from the veterinary point of view and optionally an adjuvant acceptable from the veterinary point of view.

For the expression in vivo for the purpose of producing recombinant live vaccines, the coding sequence(s) or their fragments are inserted into an appropriate expression vector under conditions allowing the expression of the polypeptide(s). As appropriate vectors, there may be used live viruses, preferably capable of multiplying in pigs, nonpathogenic for pigs (naturally nonpathogenic or rendered as such), according to techniques well known to persons skilled in the art. There may be used in particular pig herpesviruses such as Aujeszky's disease virus, porcine adenovirus, poxviruses, especially vaccinia virus, avipox virus, canarypox virus, swinepox virus. Plasmid DNAs can also be used as vectors (WO-A-90 11092, WO-A-93 19813, WO-A-94 21797, WO-A-95 20660).

The subject of the invention is therefore also the vectors and the recombinant live vaccines or plasmid vaccines (polynucleotide or DNA vaccines) thus prepared, the vaccines comprising, in addition, a vehicle or diluent acceptable from the veterinary point

of view.

The vaccine according to the invention may comprise one or more active ingredients (antigens) of one or more (2 or 3) of the circoviruses according to the invention.

The invention also provides for combining vaccination against the porcine circovirus with a vaccination against other pig pathogens, in particular those which can be associated with the PMSW syndrome. The vaccine according to the invention may therefore comprise another valency corresponding to another pig pathogen.Among these others porcine pathogens, one may cite preferably PRRS (Porcine Reproductory and Respiratory Syndrome) (WO-A-93/07898, WO-A-94/18311, FR-A-2 709 966 ; C. chareyre et al., Proceedings of the 15th IPVS Congress, Birmingham, England, 5-9 July 1998, p 139 ; incorporated therein by reference) and/or Mycoplasma hyopneumonia (EP-A-597 852, EP-A-550 477, EP-A-571 648 ; O. Martinon et al. p 157, 284, 285 and G. Reynaud et al., p 150, all in the above-referenced Proceedings of the 15th IPVS Congress ; incorporated therein by reference). Other interesting valencies are Actinobacillus pleuropneumoniae, E.coli, Atrophic Rhinitis and also Pseudorabies (Aujeszky disease), Hog cholera, Swine Influenza.

The subject of the present invention is also a method which makes it possible to induce an immune response in pigs towards circoviruses according to the invention. Its subject is in particular a method of vaccination which is effective in pigs.

This method provides for the administration to pigs, in one or more portions, of a vaccine above. It is also possible to combine several types of the above vaccines in the same vaccination protocol.

This method provides not only for administration to adult pigs, but also to young pigs or to pregnant females. The vaccination of the latter makes it possible to confer passive immunity to the newborns (maternal antibodies).

The invention also offers the possibility of diagnosing the presence of the circoviruses according to the invention in pigs. Its subject is therefore diagnostic tests and methods relating thereto using reagents which will be described below.

Knowledge of the sequences of the different circoviruses makes it possible to define common sequences which makes it possible to produce reagents capable of recognizing all the porcine circoviruses known.

Persons skilled in the art will also be able to select fragments of the sequences corresponding to regions exhibiting little or no homology with the corresponding PK/15 circovirus sequence in order to carry out a specific diagnosis.

Sequence alignments make it possible for persons skilled in the art to select a reagent in accordance with their wishes.

A first reagent consists in the DNA sequences disclosed here and their fragments, which will in particular be used as probes or primers in well-known hybridization or PCR (Polymerase Chain Reaction) techniques.

A second reagent consists in the polypeptides encoded by these sequences from the virus or expressed with the aid of a vector (see above), or synthesized by the chemical route according to conventional techniques for peptide synthesis.

A third and fourth reagent consists in respectively polyclonal and monoclonal antibodies which may be produced according to the customary techniques from the virus, the polypeptides or fragments, extracted or encoded by the DNA sequences.

These second, third and fourth reagents may be used in a diagnostic method, a subject of the invention, in which a test is carried out, on a sample of physiological fluid (blood, plasma, serum and the like) or a sample of tissue (ganglia, liver, lungs, kidneys and the like) obtained from a pig to be tested, for the presence of an antigen specific for a circovirus according to the invention, by seeking to detect either the antigen itself, or antibodies directed against this antigen.

The antigens and antibodies according to the invention may be used in any known laboratory diagnostic technique.

However, it will be preferable to use them in techniques which can be used directly in the field by the veterinary doctor, the breeder or the owner of the

animal. Persons skilled in the art have available a range of laboratory and field techniques and are therefore in the perfect position to adapt the use of this antigen and/or antibodies as diagnostic reagent(s).

The diagnostic techniques which will be preferably used within the framework of the present invention are Western blotting, immunofluoroescence, ELISA and immunochromatography.

As regards the use of immunochromatography methods, specialists can refer in particular to Robert F. Zurk et al., Clin. Chem. 31/7, 1144-1150 (1985) as well as to patents or patent applications WO-A-88/08 534, WO-A-91/12528, EP-A-291 176, EP-A-299 428, EP-A-291 194, EP-A-284 232, US-A-5 120 643, US-A-5 030 558, US-A-5 266 497, US-A-4 740 468, US-A-5 266 497, US-A-4 855 240, US-A-5 451 504, US-A-5 141 850, US-A-5 232 835 and US-A-5 238 652.

Accordingly, it is preferably sought to detect specific antibodies in the sample by an indirect test, by competition or by displacement. To do this, the antigen itself is used as diagnostic reagent, or a fragment of this antigen, conserving recognition of the antibodies. The labelling may be advantageously a labelling with peroxidase or a special labelling, preferably with colloidal gold.

It may also be desired to detect the antigen itself in the sample with the aid of a labelled antibody specific for this antigen. The labelling is advantageously as described above.

By antibody specific for the antigen which can be used in particular in competition or displacement or for the detection of the antigen itself, there is understood monoclonal or polyclonal antibodies specific for the antigen, fragments of these antibodies, preferably Fab or $F(ab)'_2$ fragments.

Another feature of the invention is the production of polyclonal or monoclonal antibodies

specific for the antigen in accordance with the invention, it being possible for these antibodies to then be used in particular as diagnostic reagent for the detection of the antigen in a sample of physiological fluid or in a tissue sample, or even for the detection of antibodies present in such a sample or specimen. The invention also includes the immunologically functional fragments of these antibodies, in particular the F(ab) and $F(ab)'_2$ fragments.

Antibodies can be prepared by the customary techniques. Reference may be made in particular to Antibodies, A Laboratory Manual, 1988, Cold Spring Harbor Laboratory, USA or to J.W. Goding, Monoclonal Antibodies: Principles and Practice, Academic Press Inc., whose contents are incorporated herein by reference.

It will be possible in particular, as is known per se, to carry out the fusion of spleen cells of mice, immunized with the antigen or with at least one of its fragments, with suitable myelomatous cells.

The subject of the invention is also a preparation, preferably pure or partially pure, or even crude, of monoclonal or polyclonal antibodies specific for the antigen, especially mouse or rabbit antibodies.

The present invention also makes it possible to determine epitopes of interest especially on the basis of the DNA sequences described here, whether epitopes of vaccinal interest or epitopes of interest in diagnosis. From the DNA sequence of the genome of the circovirus according to the invention, persons skilled in the art are in a position to determine epitopes according to known methods, for example an appropriate computer program or PEPSCAN. Epitopes are immunodominant regions of proteins and are as such regions exposed at the surface of the proteins. They can therefore be recognized by antibodies and thus be particularly used in the field of diagnosis either for the preparation of antibodies for diagnostic purposes

or for the production of corresponding peptides which can be used as diagnostic reagents.

At the very least, an epitope is a peptide having from 8 to 9 amino acids. A minimum of 13 to 25 amino acids is generally preferred.

Persons skilled in the art are therefore in a position, using one or more of these techniques as well as the other available techniques, to find epitopes for using peptides or antibodies for diagnostic purposes.

The subject of the invention is also a diagnostic kit comprising this antigen and/or polyclonal or monoclonal antibodies specific for this antigen. These are in particular diagnostic kits corresponding to the diagnostic techniques described above.

The invention will now be described in greater detail with the aid of nonlimiting exemplary embodiments, taken with reference to the drawing, in which:

Figure 1: DNA sequence of the genome of the Imp. 1011-48121 strain

Figure 2: DNA sequence of the genome of the Imp. 1011-48285 strain

Figure 3: DNA sequence of the genome of the Imp. 999 strain

Figure 4: DNA sequence of the genome of the Imp. 1010 strain

Figure 5: Alignment of the 4 sequences according to Figures 1 to 4 with the sequence of the PCV PK/15 strain

Figure 6: DNA sequence of the genome of the Imp. 999 strain as defined in the first filing in France on 3 October 1997

Figure 7: Alignments of the sequence of Figure 6 with the sequence of the PK/15 strain

Sequence listing SEQ ID

SEQ ID No: 1 DNA sequence of the genome of the Imp. 1011-48121 strain

SEQ ID No: 2 DNA sequence of the genome of the Imp. 1011-48285 strain

SEQ ID No: 3 DNA sequence of the genome of the Imp. 999 strain

SEQ ID No: 4 DNA sequence of the genome of the Imp. 1010 strain

SEQ ID No: 5 DNA sequence of the genome of the PK/15 strain

SEQ ID No: 6 DNA sequence of the genome of the Imp. 999 strain as defined in the first filing in France on 3 October 1997.

EXAMPLES

Example 1: Culture and isolation of the porcine circovirus strains:

Tissue samples were collected in France, Canada and the USA from lung and lymph nodes of piglets. These piglets exhibited clinical signs typical of the post-weaning multisystemic wasting syndrome. To facilitate the isolation of the viruses, the tissue samples were frozen at -70°C immediately after autopsy.

For the viral isolation, suspensions containing about 15% tissue sample were prepared in a minimum medium containing Earle's salts (EMEM, BioWhittaker UK Ltd., Wokingham, UK), penicillin (100 IU/ml) and streptomycin (100 µg/ml) (MEM-SA medium), by grinding tissues with sterile sand using a sterile mortar and pestle. This ground preparation was then taken up in MEM-SA, and then centrifuged at 3000 g for 30 minutes at +4°C in order to harvest the supernatant.

Prior to the inoculation of the cell cultures, a volume of 100 µl of chloroform was added to 2 ml of each supernatant and mixed continuously for 10 minutes at room temperature. This mixture was then transferred

to a microcentrifuge tube, centrifuged at 3000 g for 10 minutes, and then the supernatant was harvested. This supernatant was then used as inoculum for the viral isolation experiments.

All the viral isolation studies were carried out on PK/15 cell cultures, known to be uncontaminated with the porcine circovirus (PCV), pestiviruses, porcine adenoviruses and porcine parvoviruses (Allan G. *et al* Pathogenesis of porcine circovirus experimental infections of colostrum-deprived piglets and examination of pig foetal material. Vet. Microbiol. 1995, 44, 49-64).

The isolation of the porcine circoviruses was carried out according to the following technique:

Monolayers of PK/15 cells were dissociated by trypsinization (with a trypsin-versene mixture) from confluent cultures, and taken up in MEM-SA medium containing 15% foetal calf serum not contaminated by pestivirus (= MEM-G medium) in a final concentration of about 400,000 cells per ml. 10 ml aliquot fractions of this cell suspension were then mixed with 2 ml aliquot fractions of the inocula described above, and the final mixtures were aliquoted in 6 ml volumes in two Falcon flasks of 25 cm^2. These cultures were then incubated at +37°C for 18 hours under an atmosphere containing 10% CO_2.

After incubation, the culture medium of the semi-confluent monolayers were treated with 300 mM D-glucosamine (Cat # G48175, Sigma-Aldrich Company Limited, Poole, UK) (Tischr I. et al., Arch. Virol., 1987 **96** 39-57), then incubation was continued for an additional period of 48-72 hours at +37°C. Following this last incubation, one of the two Falcons of each inoculum was subjected to 3 successive freeze/thaw cycles. The PK/15 cells of the remaining Falcon were treated with a trypsin-versene solution, resuspended in 20 ml of MEM-G medium, and then inoculated into 75 cm^2 Falcons at a concentration of 400,000 cells/ml. The freshly inoculated flasks were then "superinfected" by

addition of 5 ml of the corresponding lysate obtained after the freeze/thaw cycles.

Example 2: Preparation of the samples of cell culture for the detection of porcine circoviruses by immunofluorescence or by *in situ* hybridization

A volume of 5 ml of the "superinfected" suspension was collected and inoculated into a Petri dish 55 mm in diameter containing a sterile and fat-free glass coverslip. The cultures in the flasks and on glass coverslips were incubated at +37°C and treated with glucosamine as described in Example 1. The cultures on glass coverslips were harvested from 24 to 48 hours after the treatment with glucosamine and fixed, either with acetone for 10 minutes at room temperature, or with 10% buffered formaldehyde for 4 hours. Following this fixing, all the glass coverslips were stored at -70°C, on silica gel, before their use for the *in situ* hybridization studies and the immunocytochemical labelling studies.

Example 3: Techniques for the detection of PCV sequences by *in situ* hybridization

In situ hybridization was carried out on tissues collected from diseased pigs and fixed with formaldehyde and also on the preparations of cell cultures inoculated for the viral isolation (see Example 2) and fixed on glass coverslips.

Complete genomic probes corresponding to the PK/15 porcine circoviruses (PCV) and to the infectious chicken anaemia virus (CAV) were used. The plasmid pPCV1, containing the replicative form of the PCV genome, cloned in the form of a single 1.7 kilo base pair (kbp) insert (Meehan B. *et al.* Sequence of porcine circovirus DNA: affinities with plant circoviruses, J. Gen. Virol. 1997, **78**, 221-227), was used as specific viral DNA source for PCV. An analogous plasmid, pCAA1, containing the 2.3 kbp replicative form of the avian circovirus CAV was used as negative control. The

respective glycerol stocks of the two plasmids were used for the production and purification of the plasmids according to the alkaline lysis technique (Sambrook J. *et al.* Molecular cloning: A Laboratory Manual. 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989) so that they are then used as templates for the preparation of the probes. The circovirus probes representative of the complete genomes of PCV and of CAV were produced from the purified plasmids described above (1 µg for each probe) and from hexanucleotide primers at random using a commercial nonradioactive labelling kit ("DIG DNA labelling kit", Boehringer Mannheim, Lewes, UK) according to the supplier's recommendations.

The digoxigenin-labelled probes were taken up in a volume of 50-100 µl of sterile water before being used for the *in situ* hybridization.

The diseased pig tissue samples, enclosed in paraffin and fixed with formaldehyde, as well as the preparations of infected cell cultures, fixed with formaldehyde, were prepared for the detection of the PCV nucleic acids according to the following technique:

Sections 5 µm thick were cut from tissue blocks enclosed in paraffin, rendered paraffin free, and then rehydrated in successive solutions of alcohol in decreasing concentrations. The tissue sections and the cell cultures fixed with formaldehyde were incubated for 15 minutes and 5 minutes respectively at +37°C in a 0.5% proteinase K solution in 0.05 M Tris-HCl buffer containing 5 mM EDTA (pH 7.6). The slides were then placed in a 1% glycine solution in autoclaved distilled water, for 30 seconds, washed twice with 0.01 M PBS buffer (phosphate buffered saline) (pH 7.2), and finally washed for 5 minutes in sterile distilled water. They were finally dried in the open air and placed in contact with the probes.

Each tissue/probe preparation was covered with a clean and fat-free glass coverslip, and then placed in an oven at +90°C for 10 minutes, and then placed in

contact with an ice block for 1 minute, and finally incubated for 18 hours at +37°C. The preparations were then briefly immersed in a 2X sodium citrate salt (SSC) buffer (pH 7.0) in order to remove the protective glass coverslips, and then washed twice for 5 minutes in 2X SSC buffer and finally washed twice for 5 minutes in PBS buffer.

After these washes, the preparations were immersed in a solution of 0.1 M maleic acid, 0.15 M NaCl (pH 7.5) (maleic buffer) for 10 minutes, and then incubated in a 1% solution of blocking reagent (Cat # 1096176, Boehringer Mannheim UK, Lewis, East Sussex, UK) in maleic buffer for 20 minutes at +37°C.

The preparations were then incubated with a 1/250 solution of an anti-digoxigenin monoclonal antibody (Boehringer Mannheim), diluted in blocking buffer, for 1 hour at +37°C, washed in PBS and finally incubated with a biotinylated anti-mouse immunoglobulin antibody for 30 minutes at +37°C. The preparations were washed in PBS and the endogenous peroxidase activity was blocked by treatment with a 0.5% hydrogen peroxide solution in PBS for 20 minutes at room temperature. The preparations were again washed in PBS and treated with a 3-amino-9-diethylcarbazole (AEC) substrate (Cambridge Bioscience, Cambridge, UK) prepared immediately before use.

After a final wash with tap water, the preparations were counterstained with hematoxylin, "blued" under tap water, and mounted on microscope glass coverslips with a mounting fluid (GVA Mount, Cambridge Bioscience, Cambridge, UK). The experimental controls included the use of a nonpertinent negative probe (CAV) and of a positive probe (PCV) on samples obtained from diseased pigs and from nondiseased pigs.

Example 4: Technique for the detection of PCV by immunofluorescence

The initial screening of all the cell culture preparations fixed with acetone was carried out by an

indirect immunofluorescence technique (IIF) using a 1/100 dilution of a pool of adult pig sera. This pool of sera comprises sera from 25 adult sows from Northern Ireland and is known to contain antibodies against a wide variety of porcine viruses, including PCV: porcine parvovirus, porcine adenovirus, and PRRS virus. The IIF technique was carried out by bringing the serum (diluted in PBS) into contact with the cell cultures for one hour at +37°C, followed by two washes in PBS. The cell cultures were then stained with a 1/80 dilution in PBS of a rabbit anti-pig immunoglobulin antibody conjugated with fluorescein isothiocyanate for one hour, and then washed with PBS and mounted in glycerol buffer prior to the microscopic observation under ultraviolet light.

Example 5: Results of the *in situ* hybridization on diseased pig tissues

The *in situ* hybridization, using a PCV genomic probe, prepared from tissues collected from French, Canadian and Californian piglets having multisystemic wasting lesions and fixed with formaldehyde, showed the presence of PCV nucleic acids associated with the lesions, in several of the lesions studied. No signal was observed when the PCV genomic probe was used on tissues collected from nondiseased pigs or when the CAV probe was used on the diseased pig tissues. The presence of PCV nucleic acid was identified in the cytoplasm and the nucleus of numerous mononuclear cells infiltrating the lesions in the lungs of the Californian piglets. The presence of PCV nucleic acid was also demonstrated in the pneumocytes, the bronchial and bronchiolar epithelial cells, and in the endothelial cells of the arterioles, the veinlets and lymphatic vessels.

In diseased French pigs, the presence of PCV nucleic acid was detected in the cytoplasm of numerous follicular lymphocytes and in the intrasinusoidal mononuclear cells of the lymph nodes. The PCV nucleic

acid was also detected in occasional syncytia. Depending on these detection results, samples of Californian pig lungs, French pig mesenteric lymph nodes, and Canadian pig organs were selected for the purpose of isolating new porcine circovirus strains.

Example 6: Results of the cell culture of the new porcine circovirus strains and detection by immunofluorescence

No cytopathic effect (CPE) was observed in the cell cultures inoculated with the samples collected from French piglets (Imp.1008 strain), Californian piglets (Imp.999 strain) and Canadian piglets (Imp.1010 strain) showing clinical signs of multisystemic wasting syndrome. However, immunolabelling of the preparations obtained from the inoculated cell cultures, after fixing using acetone and with a pool of pig polyclonal sera, revealed nuclear fluorescence in numerous cells in the cultures inoculated using the lungs of Californian piglets (Imp.999 strain), using the mediastinal lymph nodes of French piglets (Imp.1008 strain), and using organs of Canadian piglets (Imp.1010 strain).

Example 7: Extraction of the genomic DNA of the porcine circoviruses

The replicative forms of the new strains of porcine circoviruses (PCV) were prepared using infected PK/15 cell cultures (see Example 1) (10 Falcons of 75 cm^2) harvested after 72-76 hours of incubation and treated with glucosamine, as described for the cloning of the replicative form of CAV (Todd. D. *et al.* Dot blot hybridization assay for chicken anaemia agent using a cloned DNA probe. J. Clin. Microbiol. 1991, **29**, 933-939). The double-stranded DNA of these replicative forms was extracted according to a modification of the Hirt technique (Hirt B. Selective extraction of polyoma virus DNA from infected cell cultures, J. Mol. Biol. 1967, **36**, 365-369), as described by Molitor (Molitor

T.W. *et al.* Porcine parvovirus DNA: characterization of the genomic and replicative form DNA of two virus isolates, Virology, 1984, **137**, 241-254).

Example 8: Restriction map of the replicative form of the genome of the porcine circovirus Imp.999 strain.

The DNA (1-5 µg) extracted according to the Hirt technique was treated with S1 nuclease (Amersham) according to the supplier's recommendations, and then this DNA was digested with various restriction enzymes (Boehringer Mannheim, Lewis, East Sussex, UK) and the products of digestion were separated by electrophoresis on a 1.5% agarose gel in the presence of ethidium bromide as described by Todd *et al.* (Purification and biochemical characterization of chicken anemia agent. J. Gen. Virol. 1990, **71**, 819-823). The DNA extracted from the cultures of the Imp.999 strain possess a unique EcoRI site, 2 SacI sites and do not possess any PstI site. This restriction profile is therefore different from the restriction profile shown by the PCV PK/15 strain (Meehan B. *et al.* Sequence of porcine circovirus DNA; affinities with plant circoviruses, 1997 **78**, 221-227) which possess in contrast a PstI site and do not possess any EcoRI site.

Example 9: Cloning of the genome of the porcine circovirus Imp.999 strain

The restriction fragment of about 1.8 kbp generated by digestion of the double-stranded replicative form of the PCV Imp.999 strain with the restriction enzyme EcoRI was isolated after electrophoresis on a 1.5% agarose gel (see Example 3) using a Qiagen commercial kit (QIAEXII Gel Extraction Kit, Cat # 20021, QIAGEN Ltd., Crawley, West Sussex, UK). This EcoRI-EcoRI restriction fragment was then ligated with the vector pGEM-7 (Promega, Medical Supply Company, Dublin, Ireland), previously digested with the same restriction enzymes and dephosphorylated, according to standard cloning techniques (Sambrook J.

et al. Molecular cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). The plasmids obtained were transformed into an *Escherichia coli* JM109 host strain (Stratagene, La Jolla, USA) according to standard techniques. The EcoRI-EcoRI restriction fragment of the PCV Imp.999 strain was also cloned into the EcoRI site of the vector pBlueScript SK+ (Stratagene Inc. La Jolla, USA). Among the clones obtained for each host strain, at least 2 clones containing the fragments of the expected size were selected. The clones obtained were then cultured and the plasmids containing the complete genome of the Imp.999 strain were purified in a small volume (2 ml) or in a large volume (250 ml) according to standard plasmid preparation and purification techniques.

Example 10: Sequencing of a genomic DNA (double-stranded replicative form) of the PCV Imp.999 strain.

The nucleotide sequence of 2 EcoRI Imp.999 clones (clones pGEM-7/2 and pGEM-7/8) was determined according to Sanger's dideoxynucleotide technique using the sequencing kit "AmpliTaq DNA polymerase FS" (Cat # 402079 PE Applied Biosystems, Warrington, UK) and an Applied BioSystems AB1373A automatic sequencing apparatus according to the supplier's recommendations. The initial sequencing reactions were carried out with the M13 "forward" and "reverse" universal primers. The following sequencing reactions were generated according to the "DNA walking" technique. The oligonucleotides necessary for these subsequent sequencings were synthesized by Life Technologies (Inchinnan Business Park, Paisley, UK).

The sequences generated were assembled and analysed by means of the MacDNASIS version 3.2 software (Cat # 22020101, Appligene, Durham, UK). The various open reading frames were analysed by means of the BLAST algorithm available on the "National Center for

Biotechnology Information" (NCBI, Bethesda, MD, USA) server.

The complete sequence (EcoRI-EcoRI fragment) obtained initially from the clone pGEM-7/8 (SEQ ID No: 6) is presented in Figure No. 6. It starts arbitrarily after the G of the EcoRI site and exhibits a few uncertainties from the point of view of the nucleotides.

The sequencing was then optimized and the SEQ ID No: 3 (Figure 3) gives the total sequence of this strain, which was made to start arbitrarily at the beginning of the EcoRI site, that is to say the G as the first nucleotide.

The procedure was carried out in a similar manner for obtaining the sequence of the other three isolates according to the invention (see SEQ ID No: 1, 2 and 4 and Figures 1, 2 and 4).

The size of the genome of these four strains is:

Imp. 1011-48121	1767 nucleotides
Imp. 1011-48285	1767 nucleotides
Imp. 999	1768 nucleotides
Imp. 1010	1768 nucleotides

Example 11: Analysis of the sequence of the PCV Imp.999 strain.

When the sequence generated from the Imp.999 strain was used to test for homology with respect to the sequences contained in the GenBank databank, the only significant homology which was detected is a homology of about 76% (at nucleic acid level) with the sequence of the PK/15 strain (accession numbers Y09921 and U49186) (see Figure No. 5).

At amino acid level, the test for homology in the translation of the sequences in the 6 phases with the databanks (BLAST X algorithm on the NCBI server) made it possible to demonstrate a 94% homology with the open reading frame corresponding to the theoretical

replicase of the BBTV virus similar to the circoviruses of plants (GenBank identification number 1841515) encoded by the GenBank U49186 sequence.

No other sequence contained in the databanks show significant homology with the sequence generated from the PCV Imp.999 strain.

Analysis of the sequences obtained from the Imp.999 strain cultured using lesions collected from Californian piglets having clinical signs of the multisystemic wasting syndrome shows clearly that this viral isolate is a new porcine circovirus strain.

Example 12: Comparative analysis of the sequences

The alignment of the nucleotide sequences of the 4 new PCV strains was made with the sequence of the PCV PK/15 strain (Figure 5). A homology matrix taking into account the four new strains and the previous PK/15 strain was established. The results are the following:

1 : Imp. 1011-48121
2 : Imp. 1011-48285
3 : Imp. 999
4 : Imp. 1010
5: PK/15

	1	2	3	4	5
1	1.0000	0.9977	0.9615	0.9621	0.7600
2		1.0000	0.9621	0.9632	0.7594
3			1.0000	0.9949	0.7560
4				1.0000	0.7566
5					1.0000

The homology between the two French strains Imp. 1011-48121 and Imp. 1011-48285 is greater than 99% (0.9977).

The homology between the two North American strains Imp. 999 and Imp. 1010 is also greater than 99% (0.9949). The homology between the French strains and

the North American strains is slightly greater than 96%.

The homology between all these strains and PK/15 falls at a value between 75 and 76%.

It is deduced therefrom that the strains according to the invention are representative of a new type of porcine circovirus, distinct from the type represented by the PK/15 strain. This new type, isolated from pigs exhibiting the PMWS syndrome, is called type II porcine circovirus, PK/15 representing type I. The strains belonging to this type II exhibit remarkable nucleotide sequence homogeneity, although they have in fact been isolated from very distant geographical regions.

Example 13: Analysis of the proteins encoded by the genome of the new PCV strains.

The nucleotide sequence of the Imp. 1010 isolate was considered to be representative of the other circovirus strains associated with the multi-systemic wasting syndrome. This sequence was analysed in greater detail with the aid of the BLASTX algorithm (Altschul *et al.* J. Mol. Biol. 1990. **215**. 403-410) and of a combination of programs from the set of MacVector 6.0 software (Oxford Molecular Group, Oxford OX4 4GA, UK). It was possible to detect 13 open reading frames (or ORFs) of a size greater than 20 amino acids on this sequence (circular genome). These 13 ORFs are the following:

Name	Start	End	Strand	Size of the ORF (nucleotides (nt))	Protein size (amino acids (aa))
ORF1	103	210	sense	108 nt	35 aa
ORF2	1180	1317	sense	138 nt	45 aa
ORF3	1363	1524	sense	162 nt	53 aa
ORF4	398	1342	sense	945 nt	314 aa
ORF5	900	1079	sense	180 nt	59 aa
ORF6	1254	1334	sense	81 nt	26 aa
ORF7	1018	704	antisense	315 nt	104 aa
ORF8	439	311	antisense	129 nt	42 aa
ORF9	190	101	antisense	90 nt	29 aa
ORF10	912	733	antisense	180 nt	59 aa
ORF11	645	565	antisense	81 nt	26 aa
ORF12	1100	1035	antisense	66 nt	21 aa
ORF13	314	1381	antisense	702 nt	213 aa

The positions of the start and end of each ORF refer to the sequence presented in Figure No. 4 (SEQ ID No. 4), of the genome of strain 1010. The limits of ORFs 1 to 13 are identical for strain 999. They are also identical for strains 1011-48121 and 1011-48285, except for the ORFs 3 and 13:

ORF3 1432-1539, sense, 108 nt, 35aa

ORF13 314-1377, antisense, 705 nt, 234 aa.

Among these 13 ORFs, 4 have a significant homology with analogous ORFs situated on the genome of the cloned virus PCV PK-15. Each of the open reading frames present on the genome of all the circovirus isolates associated with the multisystemic wasting syndrome was analysed. These 4 ORFs are the following:

Name	Start	End	Strand	Size of the ORF (nt)	Protein size (aa)	Molecular mass
ORF4	398	1342	sense	945 nt	314 aa	37.7 kDa
ORF7	1018	704	antisense	315 nt	104 aa	11.8 kDa
ORF10	912	733	antisense	180 nt	59 aa	6.5 kDa
ORF13	314	1381	antisense	702 nt	233 aa	27.8 kDa

The positions of the start and end of each ORF refer to the sequence presented in Figure No. 4 (SEQ ID No. 4). The size of the ORF (in nucleotides = nt) includes the stop codon.

The comparison between the genomic organization of the PCV Imp. 1010 and PCV PK-15 isolates allowed the identification of 4 ORFs preserved in the genome of the two viruses. The table below presents the degrees of homology observed:

ORF Imp. 1010/ORF PVC PK-15	**Percentage homology**
ORF4/ORF1	86%
ORF13/ORF2	66.4%
ORF7/ORF3	61.5% (at the level of the overlap (104 aa))
ORF10/ORF4	83% (at the level of the overlap (59 aa))

The greatest sequence identity was observed between ORF4 Imp. 1010 and ORF1 PK-15 (86% homology). This was expected since this protein is probably involved in the replication of the viral DNA and is essential for the viral replication (Meehan *et al.* J. Gen. Virol. 1997. **78**. 221-227; Mankertz *et al.* J. Gen. Virol. 1998. **79**. 381-384).

The sequence identity between ORF13 Imp. 1010 and ORF2 PK-15 is less strong (66.4% homology), but each of these two ORFs indeed exhibits a highly conserved N-terminal basic region which is identical to the N-terminal region of the major structural protein

of the CAV avian circovirus (Meehan *et al.* Arch. Virol. 1992. **124**. 301-319). Furthermore, large differences are observed between ORF7 Imp. 1010 and ORF3 PK-15 and between ORF10 Imp. 1010 and ORF4 PK-15. In each case, there is a deletion of the C-terminal region of the ORF7 and ORF10 of the Imp. 1010 isolate when they are compared with ORF3 and ORF4 of PCV PK-15. The greatest sequence homology is observed at the level of the N-terminal regions of ORF7/ORF3 (61.5% homology at the level of the overlap) and of ORF10/ORF4 (83% homology at the level of the overlap).

It appears that the genomic organization of the porcine circovirus is quite complex as a consequence of the extreme compactness of its genome. The major structural protein is probably derived from splicing between several reading frames situated on the same strand of the porcine circovirus genome. It can therefore be considered that any open reading frame (ORF1 to ORF13) as described in the table above can represent all or part of an antigenic protein encoded by the type II porcine circovirus and is therefore potentially an antigen which can be used for specific diagnosis and/or for vaccination. The invention therefore relates to any protein comprising at least one of these ORFs. Preferably, the invention relates to a protein essentially consisting of ORF4, ORF7, ORF10 or ORF13.

Example 14: Infectious character of the PCV genome cloned from the new strains.

The plasmid pGEM-7/8 containing the complete genome (replicative form) of the Imp.999 isolate was transfected into PK/15 cells according to the technique described by Meehan B. *et al.* (Characterization of viral DNAs from cells infected with chicken anemia agent: sequence analysis of the cloned replicative form and transfection capabilities of cloned genome fragments. Arch. Virol. 1992, **124**, 301-319). Immunofluorescence analysis (see Example 4) carried out

on the first passage after transfection on noncontaminated PK/15 cells have shown that the plasmid of the clone pGEM7/8 was capable of inducing the production of infectious PCV virus. The availability of a clone containing an infectious PCV genetic material allows any useful manipulation on the viral genome in order to produce modified PCV viruses (either attenuated in pigs, or defective) which can be used for the production of attenuated or recombinant vaccines, or for the production of antigens for diagnostic kits.

Example 15: Production of PCV antigens by *in vitro* culture

The culture of the noncontaminated PK/15 cells and the viral multiplication were carried out according to the same methods as in Example 1. The infected cells are harvested after trypsinization after 4 days of incubation at 37°C and enumerated. The next passage is inoculated with 400,000 infected cells per ml.

Example 16: Inactivation of the viral antigens

At the end of the viral culture, the infected cells are harvested and lysed using ultrasound (Branson Sonifier) or with the aid of a rotor-stator type colloid mill (UltraTurrax, IKA). The suspension is then centrifuged at 3700 g for 30 minutes. The viral suspension is inactivated with 0.1% ethyleneimine for 18 hours at +37°C or with 0.5% beta-propiolactone for 24 hours at +28°C. If the virus titre before inactivation is inadequate, the viral suspension is concentrated by ultrafiltration using a membrane with a 300 kDa cut-off (Millipore PTMK300). The inactivated viral suspension is stored at +5°C.

Example 17: Preparation of the vaccine in the form of an emulsion based on mineral oil.

The vaccine is prepared according to the following formula:

- suspension of inactivated porcine circovirus: 250 ml
- Montanide® ISA 70 (SEPPIC): 750 ml

The aqueous phase and the oily phase are sterilized separately by filtration. The emulsion is prepared by mixing and homogenizing the ingredients with the aid of a Silverson turbine emulsifier.

One vaccine dose contains about $10^{7.5}$ TCID50. The volume of one vaccine dose is 0.5 ml for administration by the intradermal route, and 2 ml for administration by the intramuscular route.

Example 18: Preparation of the vaccine in the form of a metabolizable oil-based emulsion.

The vaccine is prepared according to the following formula:

- suspension of inactivated porcine circovirus: 200 ml
- Dehymuls HRE 7 (Henkel): 60 ml
- Radia 7204 (Oleofina): 740 ml

The aqueous phase and the oily phase are sterilized separately by filtration. The emulsion is prepared by mixing and homogenizing the ingredients with the aid of a Silverson turbine emulsifier.

One vaccine dose contains about $10^{7.5}$ TCID50. The volume of one vaccine dose is 2 ml for administration by the intramuscular route.

Example 19: The indirect immunofluorescence results in relation to the US and French PCV virus strains and to the PK/15 contaminant with a hyperimmune serum (PCV-T), a panel of monoclonal antibodies F99 prepared from PK/15 and a hyperimmune serum prepared from the Canadian strain (PCV-C)

	VIRUS		
	PK/15	USA	France
PCV-T antiserum	≥ 6400	200	800
PCV-C antiserum	200	≥ 6.400	≥ 6.400
F99 1H4	≥ 10 000	<100	100
F99 4B10	≥ 10 000	<100	<100
F99 2B7	≥ 10 000	100	<100
F99 2E12	≥ 10 000	<100	<100
F99 1C9	≥ 10 000	<100	100
F99 2E1	≥ 10 000	<100	<100
F99 1H4	≥ 10 000	100	<100

* Reciprocal of the last dilution of the serum or of the monoclonal antibody which gives a positive reaction in indirect immunofluorescence.